Chapter 8

Inserting and Manipulating DNA in a Nanopore with Optical Tweezers

U. F. Keyser, J. van der Does, C. Dekker, and N. H. Dekker

Summary

The translocation of small molecules and polymers is an integral process for the functioning of living cells. Many of the basic physical, chemical, and biological interactions have not yet been studied because they are not directly experimentally accessible. We have shown that a combination of optical tweezers, single solid-state nanopores, and electrophysiological ionic current detection enable deeper insight into the behavior of polymers in confinement. Here we describe the experimental procedures that are necessary to manipulate single biopolymers in a single nanopore, not only by electrical fields, but also through mechanical forces using optical tweezers.

Key words: Nanopore, Optical tweezers, DNA translocation, Biopolymers, Polymer transport, Single-molecule sensors, Single-channel recording

1. Introduction

The fabrication of the first single solid-state nanopore in an insulating membrane *(1)* pioneered the development of new techniques for nanopore fabrication and for single-molecule detection in aqueous solutions *(2)*. The ionic current flowing through a nanopore proves to be a useful tool for the label-free detection of biologically relevant polymers, ranging from DNA to proteins.

Nanopores in solid-state membranes are more robust than their biological counterparts, e.g., those found in cells. Solid-state nanopores are easily tailored in size and length to match the required characteristics for an experiment *(3)*. One intriguing idea is to use solid-state nanopores as model systems to gain

James Weifu Lee and Robert S. Foote (eds.), *Micro and Nano Technologies in Bioanalysis*,
Methods in Molecular Biology, vol. 544
DOI: 10.1007/978-1-59745-483-4_8,

a deeper understanding of polymer transport in living organisms. Prominent examples for these fundamental processes are the gene transfer between bacteria and transport of RNA and proteins through the nuclear membrane.

Here we explain the experimental techniques for mechanical manipulation of a single polymer in a solid-state nanopore *(4)*. This enables technological advances as well as new insights into basic problems in chemical physics of polymers. This novel single-molecule technique combines the high force resolution of optical tweezers with the means to detect local structures on DNA *(5)*. In a recent theoretical study, this technique was proposed for unraveling the structure of RNA molecules *(6)*. The possibility to slow down or even reverse the translocation of DNA through nanopores with optical tweezers holds potential for the detection of the primary sequence of DNA.

Nanopores are also promising building blocks for future lab-on-a-chip technologies. Together with the integration of optical techniques into microfluidic chips in combination with automation, the detection and identification of biomolecules by measuring both the ionic current and the force seems feasible.

Finally, the combination of solid-state nanopores with their biological counterparts like α-hemolysin from *Staphylococcus aureus* would circumvent common problems like the long-term stability of lipid membranes, while providing control over the nanopore shape on the single-atom level.

In the following paragraphs, we describe the experimental procedures to insert a single DNA molecule into a solid-state nanopore. Special emphasis will be given to the microfluidic cell design and suggestions for how to solve the problems we encountered during the measurements.

2. Materials

2.1. Fabrication of Solid-State Nanopores

The fabrication of solid-sate nanopores is briefly described here (excellent descriptions can be found in the literature, see, e.g., **ref.** *(7)*). In brief, a 700-nm, free-standing membrane is produced using standard semiconductor technology. Part of this membrane is thinned down by chemical etching, which leads to a 20-nm thin round silicon nitride membrane with a diameter of 5 μm. This design was chosen to make the membranes resistant to mechanical stress. The round membrane yields a diffraction pattern that is used to monitor the distance between the nanopore and optical trap (*see* 3.6).

Coating the SiN-membrane on both sides with sputtered silicon oxide (thickness 10–20 nm) facilitates wetting and reduces

sticking of colloidal particles coated with proteins. This empirical finding proved to be essential for carrying out successful experiments.

The membranes are mounted into a transmission electron microscope (TEM). The TEM is used to drill nanopores by focusing a 200- to 300-kV electron beam onto the membrane. Nanopores with <1-nm diameter can be directly drilled. The TEM shrinking method developed by Dekker and coworkers in Delft *(3)* allows for tailoring of the nanopores to the desired diameter and shape.

The nanopores are usually used within a few days of fabrication. If this is not the case, they are stored in a desiccator under vacuum. Before usage, they are treated with an oxygen plasma for 20 s to remove carbon contamination and facilitate wetting.

2.2. Optical Tweezers

The optical tweezers setup is also extensively described in the literature *(8)*. We briefly summarize the most important details, necessary for understanding the functions and proper operations (*see* **Note 1**).

The setup consists of a custom-built inverted optical microscope with a high numerical aperture water immersion objective (UPLSAPO, 60×, NA = 1.2, water immersion, Olympus, Tokyo, Japan) combined with a high-resolution patch-clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA). The water immersion objective was chosen because it is possible to trap colloidal particles up to 200 μm above the cover slip. This allows a relatively simple design of the sample cell that will be described in the next section.

The optical trap is created by overfilling the back aperture of the objective with an infrared laser (λ = 1,064 nm; P = 1.5 W; linearly polarized; CrystaLaser Inc., Reno, NV). In the focal spot, dielectric particles are confined in all three dimensions. Near the focal region, the trapping potential is harmonic, thus the restoring force F exerted on the bead is directly proportional to the distance from the center of the trap, $F = -k X$, where k is the stiffness of the trapping potential. The system represents a simple Hooke spring in all three directions. In the most general case, all three spring constants k_x, k_y, and k_z have to be characterized independently.

We use the same objective for monitoring the motion of the trapped colloid. A second, red laser (λ = 635 nm, P = 5 mW, linearly polarized, Coherent, Santa Clara, CA) is used to illuminate the bead. Decoupling of the trapping and detection lasers has certain advantages that are discussed in the literature *(9)*. The red laser is coupled into the beam path with a dichroic mirror DM1 **(Fig. 1**)that transmits in the visible range and reflects infrared (IR) light. For relative alignment to the IR laser path, its angle and position are adjusted by the mirrors M_{red}(**Fig. 1**). The red

laser is partly reflected by the trapped particle, collected by the trapping objective, and focused onto a quadrant photo detector. Spatial filtering is achieved by placing a pinhole in front of the detector. Alignment of the pinhole relative to the optical axis is crucial and has to be checked from time to time. The intervals between realignments depend on the stability of the temperature, the mechanical parts, and the pointing stability of the red laser.

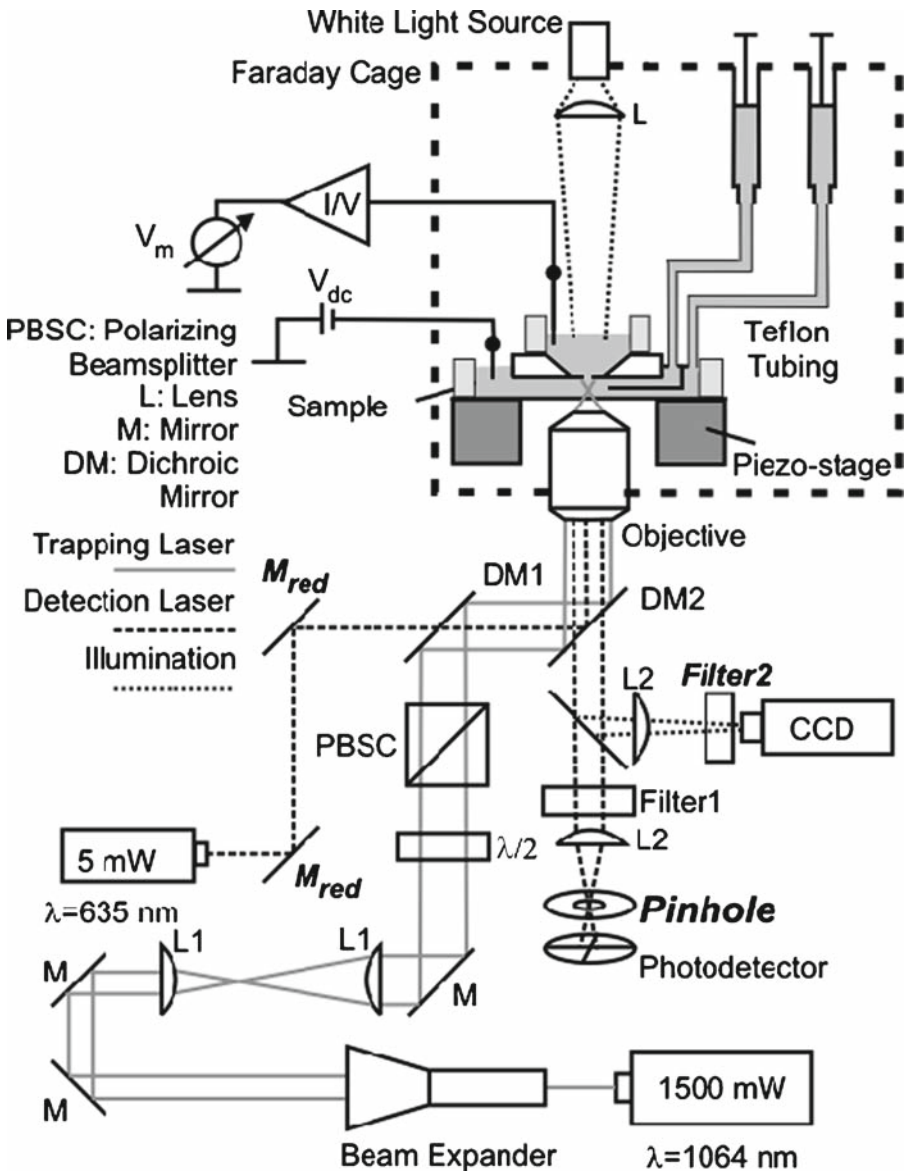

Fig. 1. Schematic of the optical tweezers setup. Two lasers (infrared and red) are coupled collinear and concentric into an objective. The objective, the head-stage amplifier, and the nanopore fluid cell are enclosed by a Faraday cage. The main elements, crucial for alignment during normal operations or maintenance, are shown in bold: two mirrors M_{red}, pinhole, and Filter2.

Alignment is preferably done in two steps to reach an optimal result as rapidly as possible. First, the IR laser path is aligned so that this trapping laser is coupled into the center of the objective. Afterward, the red laser is coupled into the IR laser path at DM1 **(Fig. 1)**. A laser power meter (Melles Griot, Carlsbad, CA, USA) and a beam profiler (TaperCamD, GenTec, Quebec, Canada) can be used to check for correct alignment behind every optical element. By inserting irises at regular intervals along the beam path, errors in the alignment can be easily identified later.

The biggest advantage of this single objective-based optical tweezers design is the ability to build a fluid cell with any size, thickness, and geometry on top of the setup. This simplifies working with solid-state nanopore chips in combination with high NA objectives.

The relative positions of the nanopore and optical trap are controlled by a three-axis piezoelectric stage (Physik Instrumente, Karlsruhe, Germany). A small Faraday cage is built around the piezoelectric stage and the microscope objective. The Faraday cage should be large enough to include all liquid handling systems to avoid electrical interference. It also contains the head stage of the patch-clamp amplifier (Axopatch 200B, Axon, Molecular Devices).

2.3. PDMS Sample Cell

The sample cell is one of the most crucial parts of the setup **(Fig. 2)**. We assemble it out of four parts. A baseplate is used as a carrier for the other parts. This allows assembling of the fluid cell containing the nanopore before mounting it on the optical tweezers. The design also facilitates testing of the nanopore and the search for leaks. A glass cover slide is used as an optical window for the microscope objective. On top, a thin layer of polydimethylsiloxane (PDMS) is used to establish a seal and create the channels for flushing in the electrolyte solutions or beads coated with DNA. A covalent bonding between the PDMS and the glass helps prevent leaks **(Fig. 2a)**. If both the PDMS and cover slide are clean, good seals are possible, allowing reuse of the PDMS layer with different cover slides. The nanopore chip is pressed onto the PDMS layer by an O-ring with a diameter of 2 mm that is held in place by a Perspex block. We apply a very thin layer of vacuum grease between the nanopore chip and the PDMS to get a good seal. The Perspex block contains the liquid connections to pump the solutions into the PDMS channel next to the objective and the top of the nanopore chip **(Fig. 2a)**. The alignment of all of the parts can be easily done by hand.

The configuration of the assembled flow cell is shown in **Fig. 2b**. The beads are flushed in beneath the nanopore, which is pressed on the PDMS layer by the O-ring in the Perspex block.

For making the PDMS layer, one has several options. The simplest option is to make a mould from aluminum that can be

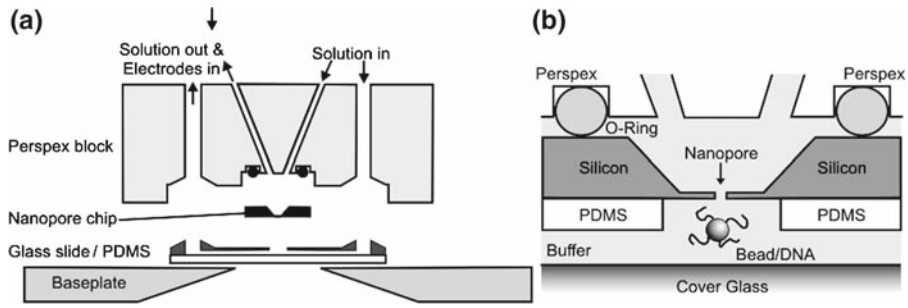

Fig. 2. Nanopore fluidic cell. (**a**) Shown are the main parts before assembly, base plate, glass slide with PDMS layer, and Perspex block, from bottom to top. The fluid inlets and outlets are shown, as well as the points where the electrodes are inserted. (**b**) The outline of the center part of the flow cell is depicted. The hole is cut into the PDMS. The diameter should be as small as possible. Please note that both figures are not drawn to scale. (The second inlet for the bottom channel, as shown in **Fig. 1**, is not depicted here for clarity. A sketch of the PDMS channels is published in **ref. (*8*)**).

reused. Modern machining tools allow accuracies of better than 0.01 mm, which is by far enough for channels with an average height of 100 μm and a total thickness of the PDMS of 150 μm. However, it is necessary to make sure that all surfaces are extremely flat to ensure a good seal. The more elaborate possibility is the use of SU8 lithography on silicon wafers. This must be weighed against the increased difficulty of obtaining usable flow cells if there is no proper equipment for photolithography available in the laboratory.

The PDMS layers are made according to the following protocol:

1. Mix PDMS 1:10 (curing agent: polymer base solution) (Sylgard 184, Dow Corning, Midland, MI).
2. It is very important to degas the PDMS before curing. This can be done with either a vacuum pump that is available in most laboratories, or with a centrifuge in combination with an ultrasonic bath.
3. Check if the mould is clean from dust particles or PDMS from previous use. Clean it if necessary.
4. After pouring the PDMS onto the mould, cure for ~10 min at 150°C.
5. Clean cover slides in an ultrasonic bath in acetone, and then in isopropanol (10 min each). Make sure the slides are well separated to ensure cleaning on both sides. Blow dry immediately before mounting in an oxygen plasma chamber (SPI Plasma-Prep II) (*see* **step 7**).
6. Check the PDMS for holes caused by air inclusions. If there are no holes in the PDMS, continue to **step 7**.

7. Treat the cover slide and the PDMS with oxygen plasma for 30 s.
8. Cut a hole into the PDMS after the film is mounted on the cover slide. This allows liquid to touch the surface of the nanopore chip. Use an injection needle that is sharpened with fine sand paper. Make sure the needle is clean by flushing it with acetone/isopropanol, wiping, and blowing it dry with nitrogen gas to remove debris from the sharpening step (*see* **Note 2**). The position of the hole can be marked with a holder, e.g., a small aluminum block where the cover slide fits into a pit with the center indicated by a small indentation. The usual diameter is >0.5 mm, depending on the injection needle. This allows easy alignment by hand.

2.4. Electrodes

Silver/silver chloride electrodes are light sensitive. To avoid the usage of these electrodes, we use platinum wires that are connected to the headstage and immersed in 1 mM potassium-ferri/ferrocyanide (Sigma Aldrich, St. Louis, MO) with 1 M KCl background solution. The solution should be stored in the dark in a refrigerator to avoid degradation. The platinum electrode configuration with the salt bridges has the advantage that no silver wire has to be chlorinated, reducing the light sensitivity compared with silver/silver chloride electrodes ***(10)***. This is important because there is intense laser irradiation from the infrared laser, which can cause electrical interference. Use small salt bridges made from agar gel with 1 M KCl to connect the sample cell with the headstage (*see* **Note 3**).

2.5. Salt Bridges

1. Heat the agar (1% in 1 M KCl) to 100°C in a microwave. Aliquot this solution into Eppendorf tubes with volumes of 1–2 mL. Let the agar harden for later use and store in the refrigerator.
2. Cut thin Teflon tubing (outer diameter <1 mm, inner diameter as large as possible) into short pieces of 10 cm in length.
3. For preparation of the salt bridge, take one agar tube and heat in a standard heating block for tubes to 100°C.
4. Using a syringe, pull the agar slowly into the Teflon tubing and afterward harden the agar by rinsing with deionized water.
5. Store the salt bridge in 1 M KCl solution in the refrigerator.

2.6. Buffers

The electrolyte solutions are made from 3 M KCl stock solutions. For the stock, potassium chloride (Sigma Aldrich ≥99.0%) is dissolved in deionized water. We use Tris-EDTA buffer solution (Sigma Aldrich) at pH 8.0 and add it to all solutions. All liquids are stored at 4°C. All solutions, excluding the ones containing DNA or beads, are filtered using 20-nm filters (Anotop

25, Whatman, Maidstone, UK) immediately before use in the setup to remove any bacteria or dust particles. The solutions are subsequently sterilized in an autoclave.

2.7. DNA and Primers

We used λ-phage DNA (Promega, Madison, WI) for the experiments. DNA primers were purchased from Biolegio, Nijmegen, The Netherlands.

3. Methods

3.1. Biotinylated DNA

The procedure to attach biotin to one end of the DNA consists basically of the hybridization and subsequent ligation of a specially designed primer to the *cos* site of the lambda DNA.
The primer sequence is: P-5′-GGGCGGCGACCTT-3′-B.

This primer is phosphorylated (P) at the 5′ end and contains a single biotin label (B) at the 3′ end.

1. First, we hybridize the primer to the *cos* site of the lambda molecule, using a 100 times molar excess of the primer. For the hybridization, use the following amounts, with concentration given in parentheses:
2. 50 μL lambda DNA (0.5 μg/μL).
3. 15 μL T4 ligase buffer (10× buffer).
4. 2 μL primer (100 μM).
5. 78 μL milliQ water.
6. Use the following hybridization program (in a polymerase chain reaction [PCR] machine) with a slow cool-down program:

   65°C for 1 h, followed by 5 min at each of the following temperatures: 63.8°C–62.5°C–61.3°C–60°C–58.8°C–57.5°C–56.3°C–55°C–53.8°C–52.5°C–51.3°C–50°C–48.8°C–47.5°C–46.3°C–45°C–43.8°C–42.5°C–41.3°C–40°C–38.8°C–37.5°C–36.3°C–35°C–33.8°C–32.5°C–31.3°C–30°C–28.8°C–27.5°C–26.3°C–25°C.
7. Cool to 4°C following the last step of the program.
8. For ligation, add 5 μL of T4 DNA ligase (New England Biolabs, Ipswitch, MA) after running the hybridization program.
9. Ligate overnight at 16°C.
10. Following the ligation, perform a phenol-chloroform extraction followed by an EtOH precipitation to remove the remaining enzymes and primers. The DNA is then dissolved

in 10 mM Tris-EDTA buffer at pH 8.0 and stored at –20°C. The typical concentration is 100–200 ng/μL.

3.2. Binding Multiple DNA Strands to Beads

1. Use 5 μL of bead suspension (e.g., 2.1-μm diameter, streptavidin coated, Polysciences, Eppelheim, Germany) to bind 5 μL of biotinylated λ-DNA (48.5 kb) at a concentration of 1 nM.
2. For washing the beads, add 100 μL of buffer (100 mM KCl, 10 mM Tris-EDTA) to beads in a 1.5-mL centrifuge tube.
3. Spin down the beads with a simple laboratory centrifuge and carefully remove the supernatant with a pipette.
4. Repeat the washing step.
5. Resuspend the beads in 5 μL of binding buffer (2 M KCl, 10 mM Tris-EDTA).
6. Add 5 μL of DNA at a concentration of 1 nM in 10 mM Tris-EDTA buffer.
7. Incubate for 15 min at room temperature. Carefully shake the vial from time to time.
8. Repeat the washing in 100 mM KCl, 10 mM Tris-EDTA.
9. Resuspend the DNA/bead constructs in measurement buffer to the desired concentration. This depends on the layout of your flow cell.
10. The concentrated DNA/beads solution can be used for 1–2 days if stored at 4°C (*see* **Note 4**).
11. Adding DNA to the beads enhances the friction coefficient (*see* **Notes 5**–7).

3.3. Assembling Flow Cell

1. Put the PDMS layer/cover slide on the base plate **(Fig. 2***)*. Apply a tiny amount of vacuum grease to the PDMS layer and mount the nanopore chip with the membrane side toward the PDMS. Press lightly to distribute the grease and do not allow contact of the membrane with the vacuum grease.
2. Hold the baseplate assembly against a light source over your head. Check if the hole in PDMS and the membrane are concentrically aligned. The membrane containing the nanopore is transparent, so a bright spot is visible when held against a light source.
3. Mount the Perspex block **(Fig. 2)** to complete the cell. M3 screws (not shown) are used to attach the Perspex block to the baseplate. Very carefully tighten the screws by hand to avoid breaking the cover slide. Because only slight overpressure is used to flush the cell, moderate tightening is sufficient to prevent leaking.

4. Fill the flow cell on both the top and bottom using a pipette. Flush slowly using a pipette to avoid creating air bubbles or breaking the membrane. Check for air bubbles before mounting the assembled cell.
5. Mount onto the optical tweezers setup.
6. Important: test all electrical connections, especially the salt bridges and the platinum electrodes.
7. Connect the tubing for flushing the cell.

3.4. Characterizing Nanopores

3.4.1. Current Voltage Characteristics

It is necessary to check the current-voltage characteristics before any experiments involving DNA translocations (*see* **Note 8**). Nanopores should exhibit a linear current voltage characteristic in the range of ±500 mV. Testing this before every experiment can save a lot of time in identifying problematic nanopores. Check if the resistance R of the nanopore differs more than a factor of two from the anticipated resistance from the basic formula for cylindrical nanopores:

$$R = \rho\left(\frac{h}{\pi r^2} + \frac{1}{2r}\right).$$

Here, r is the radius of the nanopore inferred from the TEM measurements, ρ the resistivity of the salt solution (depending on the concentration), and h is the nominal thickness of the membrane (in our case, 20–60 nm depending on the membrane, i.e., plain SiN or coated with SiO_x). Experience shows that, if the resistance happens to be higher than the expected value, there usually is a problem with the nanopore and it should be cleaned again by oxygen plasma.

3.4.2. Localizing Nanopores

The exact position of a nanopore can be easily determined by using IR laser-induced heating. The buffer absorbs part of the laser light and thus the conductivity of the aqueous salt solution increases. By scanning the laser over the membrane and simultaneously measuring the ionic current, a single nanopore is easily localized *(10)*. This is a straightforward process that can also be used to determine the spatial extension of the laser focus *(10)*. Furthermore, the laser scanning can be also used to assess the quality of the nanopores *(11)*. A typical measurement is shown in **Fig. 3a**. Depicted is a two-dimensional map of the ionic conductance of a nanopore as a function of the x and y position of the laser; high conductance regions appear as black and low conductance regions appear as white. The nanopore is in the center of the black region, which gives direct feedback about the nanopore position on the membrane.

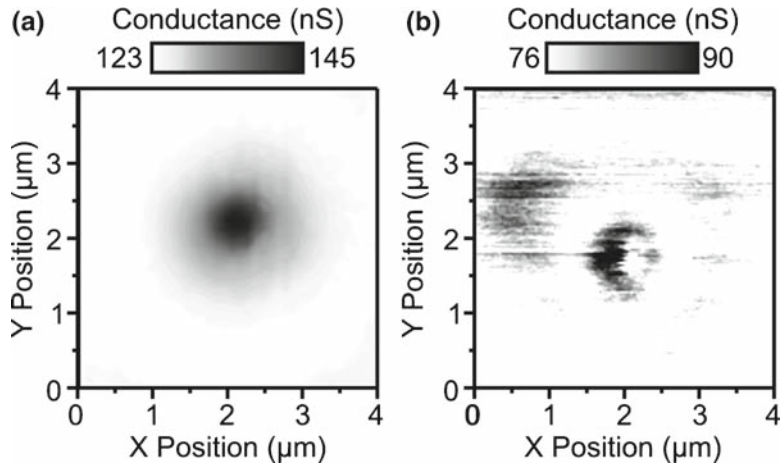

Fig. 3. (**a**) Ionic conductance as a function of laser position. The diameter of the nanopore was 11 nm. *Black* denotes high conductance and *white* low conductance. (**b**) The same measurement as (**a**) for a nanopore (10-nm diameter) with a nanobubble. The whole conductance map looks distorted and the peak at the nanopore position shows a clear substructure, indicating a bubble. Both nanopores were immersed in 1 M KCl at pH 8.0.

3.4.3. Noise in Nanopores

For a successful experiment, the number of DNA molecules inserted in the nanopore should be known at all times. This is only possible if the signal-to-noise ratio is sufficient to detect a single DNA molecule. Three major factors influence the ionic current measurements and thus the noise properties of the system, namely the electrodes, flow cell, and the nanopore itself. The electrodes were described earlier. Reducing their capacitances and access resistances is possible but not necessary because noise is determined mainly by the nanopore chip. By using a smaller flow cell with low contact resistance and small capacitance, the high-frequency noise can be reduced. The main capacitance, however, is caused by the silicon chip. The usually conductive silicon is covered by a thin insulating layer of SiN. This leads to a huge capacitance that can reach up to several 100 pF depending on the area over which the salt solution is touching the membrane. Reduction of this area is possible by adjusting the design of the flow cell (*see* **Fig. 2b**). This can be accomplished by using soft lithography techniques in PDMS. However, the main problems often arise from the solid-state nanopores. In particular, problems with wetting of nanopores were identified as sources of excessive f^{-a} (with $1<a<2$) noise in the low-frequency regime *(11)*. However, when using the two-dimensional scanning of nanopores through the laser focus, nanopores that are not properly functioning can be easily identified *(11)*. This is shown in **Fig. 3b**, in which a two-dimensional current map of a partially blocked nanopore (diameter 10 nm,

1 M KCl) is depicted. The entire scan depicts instabilities and the peak at the nanopore position exhibits a prominent substructure. This is obvious when comparing this measurement with a stable measurement depicted in **Fig. 3a**. Such unstable nanopore characteristics make controlled insertions of DNA impossible. The nanopore has to be exchanged for a new one (*see* **Notes 8–10**).

3.5. Trapping Beads in the Optical Trap

To flush solutions into the flow cell, we use 1-mL syringes that are pushed by manual micrometer screws. This has the advantage that there is no back flow after the flow is stopped because water is incompressible in this low pressure range. Of course, it is crucial to avoid any bubbles in the syringe, tubing, and the fluid cell itself to ensure proper operation (*see* **Note 10**).

1. For trapping a bead, first position the optical trap in the middle between the cover clip and the membrane containing the nanopore.
2. Start by flushing a small amount of buffer into the cell by using a syringe (**Fig. 1**).
3. Push buffer containing the bead/DNA solution (second syringe in **Fig. 1)** into the cell until you see beads flowing through the field of view.
4. Wait until one bead is trapped. This can take up to a few minutes depending on the concentration used.
5. Move the trap close to a surface (membrane or cover slip), stay typically a few microns away from the surface to avoid losing the bead because of sticking. This step avoids losing the bead because of the flushing of other buffer to get rid of the remaining beads in **step 6**.
6. Flush the rest of the beads away by flowing clean buffer solution through, using the syringe with buffer **(Fig. 1)**.
7. Characterize the trapped bead by looking at the power spectral densities in all three directions of the optical trap. Distortions of the spectrum point to problems of the alignment. This should be double checked before starting the measurements.
8. Bring the bead close to the nanopore and start inserting DNA into the nanopore.

3.6. Detecting the Distance Between Bead and Nanopore

1. Focus the laser on the nanopore by measuring the ionic current (at 50 mV) and changing the laser position. Maximize the current until the nanopore is in focus without a trapped bead.
2. Using the CCD camera, record images of the diffraction pattern caused by the nanopore at increasing distances between the focal point of the laser and the nanopore. Three examples

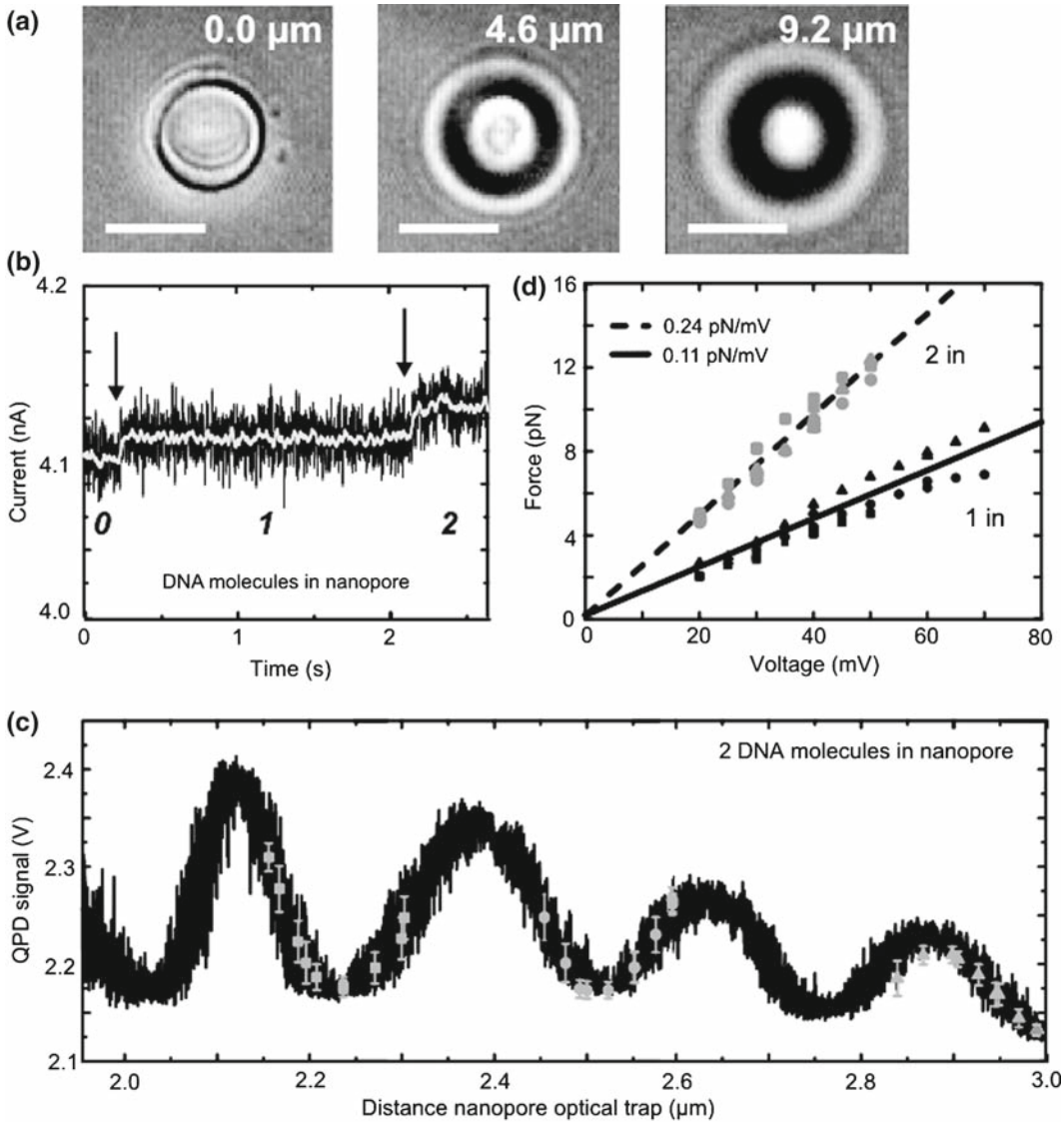

Fig. 4. Force measurements on DNA in a 80-nm nanopore. (**a**) Video images of the membrane containing the nanopore. The diffraction pattern depends on the distance between focus and membrane. The scale bar is 5 µm. (**b**) Ionic current as a function of time showing the stepwise increase in signal indicating insertion of a single DNA molecule in the nanopore (indicated by *arrows*). The *black* data is filtered at 1 kHz. The *light grey* data is filtered by a moving average over 20 points. (**c**) Calibration curve (*black*) of the total intensity on the quadrant photo detector (QPD) as a function of distance between the nanopore and optical trap. *Points* indicate force measurements with the two DNA molecules from (**a**) in the nanopore. (**d**) Comparison of force for one and two molecules in this nanopore. The force doubles when two DNA molecules are inserted. All measurements were done in 33 mM KCl, pH 8.0.

are shown in **Fig. 4a**. Use a step size of 300 nm and 31 steps to get a calibration up to 9 µm from the nanopore. This is similar to the process used, e.g., in magnetic tweezers; details can be found in the literature *(12)*. This technique works per-

fectly if the diameter of the membrane is at least a factor of two larger than the bead diameter.

3. Return the laser focal point to the nanopore. Increase the distance between the focal point and the nanopore to 2, 4, and 6 μm, respectively. At each position, check that an accurate position measurement can be determined from the diffraction pattern as explained in the literature *(12)*. In brief, the positions of the diffraction rings are compared with the recorded diffraction pattern (*see* **step 2**). Intermediate position values are interpolated.

3.7. Getting DNA into the Nanopore

1. Depending on the length of the DNA, insertion of DNA is most likely if the distance between the bead surface and the nanopore is smaller than the radius of gyration of the DNA. Move the surface of the bead coated with DNA to within 1 μm of the nanopore if λ-DNA is used.
2. Apply a voltage with polarity such that DNA is pulled into the nanopore, i.e., positive voltage at the top chamber in **Fig. 1**.
3. Monitor the ionic current.
4. When the current changes, one or more DNA molecules are inserted into the nanopore. The current change indicates the number of DNA molecules in the nanopore *(5)*. The ionic current can increase or decrease depending on salt concentration *(5)*. Typical data for DNA entering a nanopore at 33 mM KCl is shown in **Fig. 4b**. DNA entering the nanopore can be clearly observed.
5. If too many molecules are trapped, reverse the voltage to drive the DNA out of the nanopore. Increase the distance between bead and nanopore and/or reduce the voltage to avoid multiple captures. This is especially important when using large nanopores with diameter >20 nm, as can be observed by the short interval between captures of less than 2 s observed in **Fig. 4b**.
6. After successful capture, increase the distance between nanopore and bead to more than four times the radius of gyration of the DNA to avoid further captures of DNA (*see* **Note 11**).
7. Start experiments.

3.8. Force Measurements

1. Reduce the voltage to 10–20 mV to keep the DNA in the nanopore.
2. Increase the distance between the optical trap and the nanopore using the piezoelectric stage and monitor the total power at the quadrant photo detector. This will lead to data similar to the black curve in **Fig. 4c**.
3. Increase the voltage stepwise to 100 mV and record the power at the detector for several seconds.

4. Repeat the experiment at the same position at least twice. Always return to the starting voltage to check for sticking of the DNA to the nanopore wall or some other surface (*see* **Note 12**).
5. Average the detected power at every voltage step and compare this with the measured retraction curve that is shown in **Fig. 4c** by the black line. Comparison of the averaged signals with the calibration curves (points in **Fig. 4c**) returns the position and force values at this voltage. The result for two DNA molecules in 33 mM KCl is shown in **Fig. 4d**. The scatter of the points reflects the error in the position detection. The dashed line is a fit through the data taken at three different distances between nanopore and optical trap. For comparison, data for one DNA molecule in the same nanopore is also shown in **Fig. 4c**. The gradient or force/voltage (0.24 ± 0.01 pN/mV) on two DNA molecules is twice as high as for a single DNA molecule (0.11 ± 0.01 pN/mV) within experimental errors (*see* **Note 13**).

3.9. Conclusions

With the above-described experimental procedures, single DNA molecules can be readily inserted into solid-state nanopores. All of the common pitfalls we encountered are described in great detail in the above text. For an experienced master student, it is possible to mount two nanopores per day on the setup, including all cleaning steps, provided the nanopores are already available. It is also worth mentioning that we had experiments where a single nanopore worked for several days in a row. Reusing nanopores is possible after thorough cleaning. This is always determined by how clean the solutions are. In conclusion, we hope that this chapter is helpful in inserting single molecules in nanopores and enables exciting new experiments in the coming years.

4. Notes

In this section, we review the most common problems encountered during the experiments, with their respective diagnoses and solutions.

1. Bad alignment of the optical trap can be easily identified by observing the power spectral density of the bead as a function of x-, y-, and/or z-position. If it is not of Lorentzian form (dropping off with $1/f^2$ at frequencies above f_c), something is wrong. Start by checking alignment of the red laser relative to the IR laser. Trap a bead, remove the laser line filter (Filter 2, **Fig. 1**) in front of the camera, and check if the bead is

uniformly illuminated by the red laser. If this is not the case, realign the red laser with the two mirrors (M_{red}, **Fig. 1**). Next, it is necessary to check the alignment of the pinhole relative to the red laser. Do this also with a trapped bead. Afterward, look at the power spectrum. If it is normal, start to measure. Otherwise, test if the bead is the problem. Trap a new bead and look at the spectrum again. If the problem persists, repeat the red laser/pinhole alignment steps. When you are sure that this is not the problem, realign the IR laser. Important: only move the mirrors of the IR path (labeled M, **Fig. 1**) if you are absolutely sure that there is indeed a problem.

2. Clean PDMS layers are crucial. Cleaning in methanol and with ultrasound removes any remaining particles from previous experiments.
3. No ionic current through nanopore: If there are problems with the electrical signal, first test the electrodes. Start with the platinum wires and salt bridges. Next, check for bubbles in the PDMS or Perspex channels. If the wires do not work, clean them again. If the salt bridges have high resistance, cut off short pieces on both ends with a sharp scalpel or use a new salt bridge. Bubbles can normally be seen by eye and flushed away. This has to be done carefully to avoid breaking the membrane containing the nanopore.
4. To ensure long-term stability of the DNA, only use autoclaved pipette tips and wear gloves at all times. Contamination of the DNA stock can lead to rapid degradation. When the DNA is stored at 4°C, it is possible to use the same DNA for more than half a year with the same results.
5. A large amount of DNA on the bead will increase the friction coefficient of the bead. Because the trap stiffness is not influenced by the DNA on the bead, a reduction of the corner frequency *(9)* should be observed. The corner frequency in the power spectrum for a bead with a lot of DNA is usually half compared with a bead of similar size without DNA.
6. If a fluorescence setup is available, there is a convenient way to see DNA on the beads. Stain the DNA with YoYo-1 (Invitrogen, Carlsbad, CA) after performing the binding procedure and observe the beads with a fluorescent microscope to determine if DNA is bound. This can avoid much wasted time in case the binding does not work. It can also be used for fast determination of whether the DNA stock is still intact.
7. The exact concentration of beads and DNA varies from batch to batch. Thus, the parameters have to be slightly adjusted from time to time.
8. Only after excluding the problems described in **Note 3** should possible problems with the nanopore be considered.

9. Usually, in our experiments, a nanopore that shows high $1/f$ noise, randomly changing conductance levels or total blockades of ionic currents should be replaced and the experiment should be restarted. Trying to insert DNA controllably in such a situation is not possible.
10. Bubble formation in the buffer can be prevented by degassing before the experiments. This can be done by placing the solution into a vacuum flask and removing the gas with a vacuum pump. Ultrasound also helps to remove dissolved gases. It is best to combine both procedures.
11. If DNA seems not to enter the nanopore, first check the power spectrum of the bead in the trap. If f_c of the trapped bead is close to f_c of beads without DNA, find a bead with more DNA (lower f_c). Afterward, increase the voltage and decrease the distance. If the surface is nonsticky and the bead is densely coated with DNA, the bead can be pressed against the membrane without getting stuck. If this does not help and all of the beads have a high f_c, test the DNA (*see* **Notes 5** and **6**). If the DNA is okay, the next most likely explanation is that the nanopore has a high surface charge. Use larger nanopores and high salt concentrations to get DNA into the nanopore.
12. Sticking of the DNA/beads to the membranes is one of the most serious problems because under normal circumstances this implies that the experiment is over. The odds are minimized by using glass surfaces (sputtered SiO_x). There is also the possibility of adding a tiny amount of Tween to the solutions. This tends to help.
13. Check the alignment of the IR and red laser on a regular basis. Otherwise, results from the force measurements can be totally incorrect.
14. Avoid flushing the solutions with too much force because this can lead to rupture of the DNA ***(13)*** on the beads or breaking of the membranes ***(14)***.

Acknowledgments

We thank Peter Veenhuizen, Stijn van Dorp, Bernard Koeleman, Ya-Hui Chen, and Suzanne Hage for making the biotinylated lambda-DNA, and Bernadette Quinn for help with electrochemical questions. Ralph Smeets, Diego Krapf, and Meng-Yue Wu fabricated the nanopores. Stijn van Dorp and Bernard Koeleman are especially acknowledged for obtaining some of the data presented here. Financial support of FOM and NWO is gratefully acknowledged.

References

1. Li, J., Stein, D., McMullan, C., Branton, D., Aziz, M. J., and Golovchenko, J. A. (2001). Ion-beam sculpting at nanometre length scales. *Nature* **412**, 166–169
2. Dekker, C. (2007). Solid-state nanopores. *Nature Nanotechnology* **2**, 209–215
3. Storm, A. J., Chen, J. H., Ling, X. S., Zandbergen, H. W., and Dekker, C. (2003). Fabrication of solid-state nanopores with single-nanometre precision. *Nature Materials* **2**, 537–540
4. Keyser, U. F., Koeleman, B. N., van Dorp, S., Krapf, D., Smeets, R. M. M., Lemay, S. G., Dekker, N. H., and Dekker, C. (2006). Direct force measurements on DNA in a solid-state nanopore. *Nature Physics* **2**, 473–477
5. Smeets, R. M. M., Keyser, U. F., Krapf, D., Wu, M. Y., Dekker, N. H., and Dekker, C. (2006). Salt dependence of ion transport and DNA translocation through solid-state nanopores. *Nano Letters* **6**, 89–95
6. Gerland, U., Bundschuh, R., and Hwa, T. (2004). Translocation of structured polynucleotides through nanopores. *Physical Biology* **1**, 19–26
7. Krapf, D., Wu, M. Y., Smeets, R. M. M., Zandbergen, H. W., Dekker, C., and Lemay, S. G. (2006). Fabrication and characterization of nanopore-based electrodes with radii down to 2 nm. *Nano Letters* **6**, 105–109
8. Keyser, U. F., van der Does, J., Dekker, C., and Dekker, N. H. (2006). Optical tweezers for force measurements on DNA in nanopores. *Review of Scientific Instruments* **77**, 105105
9. Visscher, K. and Block, S. M. (1998). Versatile optical traps with feedback control. *Molecular Motors and the Cytoskeleton, Part B* **298**, 460–489
10. Keyser, U. F., Krapf, D., Koeleman, B. N., Smeets, R. M. M., Dekker, N. H., and Dekker, C. (2005). Nanopore tomography of a laser focus. *Nano Letters* **5**, 2253–2256
11. Smeets, R. M. M., Keyser, U. F., Dekker, N. H., and Dekker, C. (2006). Nanobubbles in solid-state nanopores. *Physical Review Letters* **97**, 088101
12. Strick, T. R., Allemand, J. F., Bensimon, D., Bensimon, A., Croquette, V. (1996). The elasticity of a single supercoiled DNA molecule. *Science* **271**, 1835–1837
13. Davison, P. F. (1959). The effect of hydrodynamic shear on the deoxyribonucleic acid from T2 and T4 bacteriophages. Proceedings of the national Academy of Sciences USA **45**, 1560–1568
14. Tong, H. D., Jansen, H. V., Gadgil, V. J., Bostan, C. G., Berenschot, E. van Rijn, C. J. M., and Elwenspoek, M. (2004). Silicon Nitride Nanosieve Membrane. Nano Letters **4**, 283–287